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Complete Nucleotide Sequences of Hepatitis B Virus Genomes Associated With Epidemic Fulminant Hepatitis

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Pre-core/core mutants are frequently observed in patients with fulminant hepatitis. To investigate the extent of molecular characteristics of hepatitis B virus (HBV) genomes implicated in the development of fulminant hepatitis, full-length HBV genomes were sequenced directly from sera of two patients with epidemic fatal fulminant hepatitis, after amplification by the polymerase chain reaction. These two genomes, of 3215 nucleotides, were 99.6% identical, indicating that a common source of HBV potentially caused fulminant hepatitis. Thirty unique nucleotide mutations were commonly found in the two entire HBV genomes. Three were located in the stem-loop structure, changing this element to a more stable structure. Twenty-five unique amino acid substitutions were found in each open reading frame, except for the X and pre-surface 2 genes. One was located in the pre-surface 1 gene; two were in the surface gene; three were in the pre-core gene, including codons 28 (tryptophan to stop codon) and 29 (glycine to aspartic acid); eight were in the core gene; and 11 were in the polymerase gene. The pre-core mutations at codons 28 and 29 were common to the two HBV strains reported previously in patients with epidemic fulminant hepatitis. Thus, HBV genomes associated with epidemic fatal fulminant hepatitis have numerous unique mutations, located mainly in the polymerase gene, as well as the pre-core/core gene, including mutations in the stem-loop structure of the pregenome encapsidation signal sequence. These mutations may be associated with the development of fulminant hepatitis.

KEY WORDS: direct sequencing, fulminant hepatitis, hepatitis B, polymerase chain reaction

INTRODUCTION

Hepatitis B virus (HBV) causes a variety of clinical manifestations in acutely infected individuals ranging from self-limited acute hepatitis to fulminant hepatitis. Viral mechanisms of the development of fulminant hepatitis, however, are poorly understood. Although pre-core mutants, which cannot translate the pre-core messenger RNA to hepatitis B e antigen (HBeAg), are detected frequently in patients with fulminant hepatitis [Omata et al., 1991], these mutants are not necessarily found in this setting [Carman et al., 1991]. On the other hand, in patients with fulminant or severe hepatitis, various mutations were found in the core gene [Ehata et al., 1993] that has been shown to code epitopes for cytotoxic T cells (CTL) [Ferrari et al., 1987]. These mutations, however, are also found in hepatitis B e antibody-positive asymptomatic carriers.

Epidemic fulminant hepatitis B in multiple contacts of a single HBV strain provides a rare opportunity to investigate the genetic basis of fulminant hepatitis B, since host factors, including immune responses or human leukocyte antigen (HLA) haplotypes, can be excluded for consideration in this setting. Only two full-length HBV genomes were reported previously in patients with epidemic fulminant hepatitis B. Ogata et al. [1993] determined the entire nucleotide sequence of an HBeAg-minus HBV genome of genotype C (strain HT) that induced fulminant hepatitis in recipients, and identified many unique nucleotide mutations and amino acid substitutions, especially in the core promoter region. Hasegawa et al. [1994] also reported the whole HBV genome of genotype D (strain FH) that was

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The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence database with the following accession numbers: D50521 and D50522.

TABLE I. Clinical Characteristics of the Two Patients With Epidemic Fatal Fulminant Hepatitis on Admission

Case	Age/sex	Alanine aminotransferase (IU/L)	Prothrombin time (%)	HBsAg	anti-HBs	anti-HBc
1	82/M	4792	20	+	+	+
2	66/M	6615	29	+	−	+

associated with epidemic fulminant hepatitis in Israel. Although these findings in patients with epidemic fulminant hepatitis B suggest that some virus-specific factors may contribute to the development of fulminant hepatitis, genetic characteristics are still not fully clear.

The aim of the present study was to determine complete nucleotide sequences of HBV genomes obtained from patients with epidemic fatal fulminant hepatitis B, and to investigate molecular characteristics implicated in the development of fulminant hepatitis.

MATERIALS AND METHODS

Patients

In September 1994, four Japanese patients contracted successively fatal fulminant hepatitis B. All of these patients had been treated by maintenance hemodialysis in the same clinic until the onset of fulminant hepatitis. None were known drug abusers or had received blood or blood product transfusion during the 6 months before onset. None had any other causes of liver dysfunction including viral hepatitis (except hepatitis B), or nonviral diseases, such as hereditary liver diseases, excessive alcohol intake, or hepatotoxic drug administration. They had been negative for hepatitis B surface antigen (HBsAg) before the fulminant hepatitis, indicating that they were not HBV carriers. The Investigation Committee of the Tokyo Metropolitan Bureau of Public Health announced that this epidemic fulminant hepatitis was recognized as a nosocomial infection caused by a common source. All four patients had similar clinical courses, with clinical evidence of massive liver necrosis and rapid deterioration to hepatic encephalopathy and death. For the present study, sera at the onset were available from two of these patients. Clinical characteristics of these two patients are shown in Table I.

Polymerase Chain Reaction (PCR) Amplification of HBV-DNA

Serum samples obtained sequentially from the patients were stored at −70°C until use. HBV-DNA was extracted from the sera by a sodium hydroxide procedure [Kaneko et al., 1989]. Briefly, 10 μl of serum were treated with 0.2 N sodium hydroxide in a volume of 20 μl at 37°C for 30 minutes. After neutralization with hydrochloric acid, a 1 μl aliquot was then amplified by the hot start PCR without cloning in a 50 μl mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 10 pmole of each "outer" PCR primer, 200 μM each dNTP, 0.01% gelatin, and 1 unit of Taq polymerase (Promega, Madison, WI) for 40 cycles. Each cycle consisted of denaturing at 94°C for 1 minute, annealing of the primers at 55°C for 1 minute, and extension at 72°C for 1 minute. Then, 1 μl of the first PCR products of each fragment was transferred to the second PCR with the "nested" 5′ and 3′ primers. pUC/M13-forward (18 nucleotides [nt]), T7 promoter (20 nt), or pUC/M13-reverse (18 or 22 nt) sequencing primer sequence was attached to the 3′ terminus of the 5′- and 3′-nested primers, respectively, to facilitate direct sequencing in an automated DNA sequencer. The second PCR was carried out under the same reaction condition as the first PCR, except for primers. The second PCR products were electrophoresed through agarose gel and visualized under ultraviolet light. Negative controls were included in each PCR round, and false-positive results were avoided by the strict application of contamination-prevention guidelines.

Direct Sequencing of PCR-Amplified DNA

After residual dNTPs and primers were removed using a centrifugation filtration column (Suprec-02, Takara, Kyoto, Japan), nucleotide sequences of the PCR products were determined directly using the Taq DyeDeoxy Terminator Cycle sequencing kit with an automated DNA sequencer model 373A (Applied Biosystems Japan, Tokyo, Japan). In brief, 7 μl of the purified second PCR products were mixed with 3.2 pmole of sequencing primers, 1 μl of each DyeDeoxy Terminator (A, G, C, T), 1 μl of the dNTP mixture (750 μM dITP, 150 μM dATP, 150 μM dTTP, and 150 μM dCTP), 4 units of AmpliTaq DNA polymerase, and 4 μl of the reaction buffer (400 mM Tris-HCl, 10 mM $MgCl_2$, and 100 mM $(NH_4)_2SO_4$, pH 9.0), in a volume of 20 μl. The sequencing primers were pUC/M13-forward or T7 promoter primer for the sense strand, and pUC/M13-reverse primer (18 or 22 nt) for the anti-sense strand. Cycle sequencing reaction was carried out for 25 cycles, each cycle consisting of a rapid thermal ramp to 96°C, 96°C for 30 seconds, rapid thermal ramp to 50°C, 50°C for 15 seconds, rapid thermal ramp to 60°C, and 60°C for 4 minutes. Excess of DyeDeoxy Terminators was removed from the completed reaction mixture using a Quick spin column (Boehringer Mannheim GmbH, Mannheim, Germany). Samples were dried in a vacuum centrifuge, dissolved in a loading buffer (5 μl deionized formamide and 1 μl of 50 mM EDTA, pH 8.0), and loaded onto an Applied Biosystems 373A DNA sequencer according to the manufacturer's instructions.

TABLE II. Nucleotide Sequences of the Primers for PCR*

Sequences	Nucleotides
First PCR primers	
TGAGTGGGAGGAGCTGGGGGAGGA	1727–1750 sense
GGTCCCACAAATTGCTTACATCTA	2576–2599 anti-sense
GGGAAACTTTACTGGGCTTTATTC	2474–2497 sense
TAGAAAATTGAGAGAAGTCCACCA	257–280 anti-sense
CCTAGGACCCCTGCTCGTGTTACA	178–201 sense
AAGTTGGCGAGAAAGTGAAAGCCT	1085–1108 anti-sense
GGCCTATTGATTGGAAAGTATGTC	968–991 sense
GGAAAAAGTTGCATGGTGCTGGTG	1804–1826 anti-sense
ACGTCCGCATGGAGACCACCG	1601–1620 sense
GGAAAGAAGTCAGAAGGCAAA	1954–1974 anti-sense
Second PCR primers	
TGTAAAACGACGGCCAGTGATTAGGTTAAAGGTCTTTG	1751–1770 sense
CAGGAAACAGCTATGACCGTGCGAATCCACACTCCAAA	2264–2283 anti-sense
TGTAAAACGACGGCCAGTCTTACTTTTGGAAGAGAGAC	2222–2241 sense
TCACACAGGAAACAGCTATGACAAGGTCCTCCTGCAAATGAA	2551–2570 anti-sense
TGTAAAACGACGGCCAGTTAATCCTGAGTGGCAAACTC	2516–2535 sense
TCACACAGGAAACAGCTATGACCCGAATGCTCCCGCTCCAAC	3019–3038 anti-sense
TGTAAAACGACGGCCAGTCAAACAATCCAGATTGGGAC	2960–2979 sense
TCACACAGGAAACAGCTATGACCGAGTCTAGACTCTGTGGTA	237–256 anti-sense
TGTAAAACGACGGCCAGTGGCGGGGTTTTTCTTGTTGA	202–221 sense
TCACACAGGAAACAGCTATGACATACATGCATATAAAGGCAT	1054–1073 anti-sense
TGTAAAACGACGGCCAGTCGGGACCATGCAAGACCTGC	507–526 sense
TGTAAAACGACGGCCAGTGGTCTTTTGGGCTTTGCTGC	1003–1022 sense
TCACACAGGAAACAGCTATGACCGCAGACCAATTTATGCCTA	1784–1803 anti-sense
TGTAAAACGACGGCCAGTCCTAGCAGCTTGTTTTGCTC	1281–1300 sense
TCACACAGGAAACAGCTATGACGAGAAACGGACTGAGGCCCA	647–666 anti-sense
TAATACGACTCACTATAGGGGAGACCACCGTGAACGCCCA	1611–1630 sense
TCACACAGGAAACAGCTATGACGAGAGTAACTCCACAGAAGCT	1930–1950 anti-sense

*Underlined sequences are the sequencing primer sequences attached to 3′ terminus of the second PCR primers. Nucleotide positions are numbered from the unique *Eco* RI site.

Oligonucleotide Primers

The PCR and sequencing primers were synthesized by a DNA synthesizer model 391 (Applied Biosystems Japan). In order to cover the whole HBV genomes, five fragments of HBV-DNA were amplified for the first-stage PCR, and nine fragments for the second-stage PCR, with the amplifications overlapping each other. The nucleotide sequences of the primers are listed in Table II.

Comparison of the Nucleotide Sequences With Previously Reported HBV Genomes

In order to determine the reference sequence of full-length HBV genomes, multiple alignments of the nucleotide sequences were made and amino acid residues of 20 reported full-length HBV clones previously that were unrelated to fulminant hepatitis were deduced [Galibert et al., 1979; Valenzuera et al., 1980; Fujiyama et al., 1983; Ono et al., 1983; Kobayashi and Koike, 1984; Bichko et al., 1985; Okamoto et al., 1986, 1987, 1988; Sastrosoewignjo et al., 1987; Rho et al., 1989; Loncarevic et al., 1990; Takemura et al., 1990]. "Consensus" sequences were considered to be those that were observed in more than 70% at each nucleotide and amino acid position in the same subtype, and "unique mutations" to be those not observed in any reported clones at each position.

RESULTS

Nucleotide sequences of the full-length HBV genomes were determined successfully in both patients. Both genomes were composed of 3,215 bases in length and were classified into subtype *adw* and genotype B [Okamoto et al., 1988]. Both genomes shared 99.6% identical nucleotide sequences, and three amino acid differences were found in each of the surface gene (codon 42, leucine and proline; codon 129, glutamic acid and arginine; and codon 130, glycine and asparagine) and the polymerase gene (codon 463, serine and proline; codon 572, asparagine and histidine; and codon 484, lysine and arginine). Two of the three differences in the surface gene were located in the *a* determinant region (codons 129 and 130).

In comparison with the consensus nucleotide sequence deduced from the literature as described in the Materials and Methods, 30 unique nucleotide mutations, including 5 silent mutations, were common to the two samples analyzed (Table III, Fig. 1). In the regulatory element of HBV genomes, three mutations were observed in the stem-loop structure (Fig. 2), and two were observed in the enhancer I-X promoter region. No mutations were found in the core-promoter, surface promoter I, and surface promoter II regions. In comparison with the consensus amino acid sequence, 25 unique

TABLE III. Unique Nucleotide and Deduced Amino Acid Mutations Isolated From the Patients With Epidemic Fatal Fulminant Hepatitis B*

Nucleotide mutations			Amino acid mutations			
nt	Consensus	Present case	Codon position		Consensus	Present case
223	A	C	P (pol/RT)	378	Lys	Gln
279	T	C[a]	Surface	42	Leu	Pro[a]
286	A	C	P (pol/RT)	399	Ser	His
467	C	G	Surface	105	Pro	Ala
			P (pol/RT)	459	Ala	Gly
478	T	C[b]	P (pol/RT)	463	Ser	Pro[b]
540	A	G[a]	Surface	129	Gln	Arg[a]
542	G	A[a]	Surface	130	Gly	Asn[a]
543	G	A[a]	P (pol/RT)	484	Arg	Lys[a]
544	A	C	P (pol/RT)	485	Asn	His
777	T	C	Surface	208	Ile	Thr
805	A	C[a]	P (pol/RT)	572	Asn	His[a]
929	T	C	P (pol/RT)	613	Leu	Pro
992	A	C[c]	P (pol/RT)	634	Gln	Pro
996	A	C[c,d]				
1536	T	C[d]				
1847	T	C[e]	Pre-C	12	Cys	Arg
1896	G	A[e]	Pre-C	28	Trp	stop[f]
1899	G	A[e]	Pre-C	29	Gly	Asp[f]
1961	T	G	Core	21	Ser	Ala
1983	G	A	Core	28	Arg	His
1984	A	T				
2063	C	A	Core	55	Leu	Ile
2108	A	T	Core	70	Thr	Ser
2174	A	C	Core	92	Asn	His
2222	C	A	Core	108	Leu	Ile
2430	A	G	Core	177	Gln	Arg
2431	A	G	P (5′TBP)	42	Asn	Gly
2435	C	A[d]				
2441	T	C	Core	181	Ser	Pro[g]
2510	T	G[d]				
2568	A	C	P (5′TBP)	88	Ile	Leu
2684	T	G	P (5′TBP)	126	Asp	Glu
2699	T	C[d]				
2736	A	G	P (5′TBP)	144	Thr	Ala[h]
2807	A	G[a,d]				
2857	T	C	P (Spacer)	184	Leu	Ser
2858	G	C	Pre-S1	4	Trp	Pro

*nt denotes nucleotide position numbered from the unique *Eco*RI site. 5′TBP denotes 5′ terminal binding protein. pol/RT denotes DNA polymerase-reverse transcriptase. Amino acid residues are represented by triple-letter codes.
[a]The mutation only found in patient 1, who was positive for anti-HBs antibody.
[b]The mutation only found in patient 2, who was negative for anti-HBs antibody.
[c]The mutation that was located in enhancer I-X promoter element.
[d]Denotes silent mutation.
[e]The mutation that was located in encapsidation signal ϵ.
[f]The same mutation was previously reported in strains HT [Ogata et al., 1993] and FH [Hasegawa et al., 1994].
[g]The same mutation was previously reported in strain HT.
[h]The same mutation was previously reported in strain FH.

amino acid substitutions were found in the entire HBV genomes. One substitution was found in the pre-surface 1 gene; two were found in the surface gene; three were found in the pre-core gene, including substitutions in codons 12 (cysteine to arginine), 28 (tryptophan to stop codon), and 29 (glycine to aspartic acid); eight were found in the core gene; and 11 were found in the polymerase gene. Among 11 mutations in the polymerase gene, 4 were in the 5′ terminal protein domain, 1 was in the spacer domain, and 6 were in the DNA polymerase-reverse transcriptase domain; but none in the ribonuclease H domain. No unique substitutions were found in the X gene.

Comparison of these unique amino acid mutations with the previously reported two full-length HBV sequences associated with epidemic fulminant hepatitis, strains HT [Ogata et al., 1993] and FH [Hasegawa et al., 1994], is shown in Figure 3. Only two substitutions

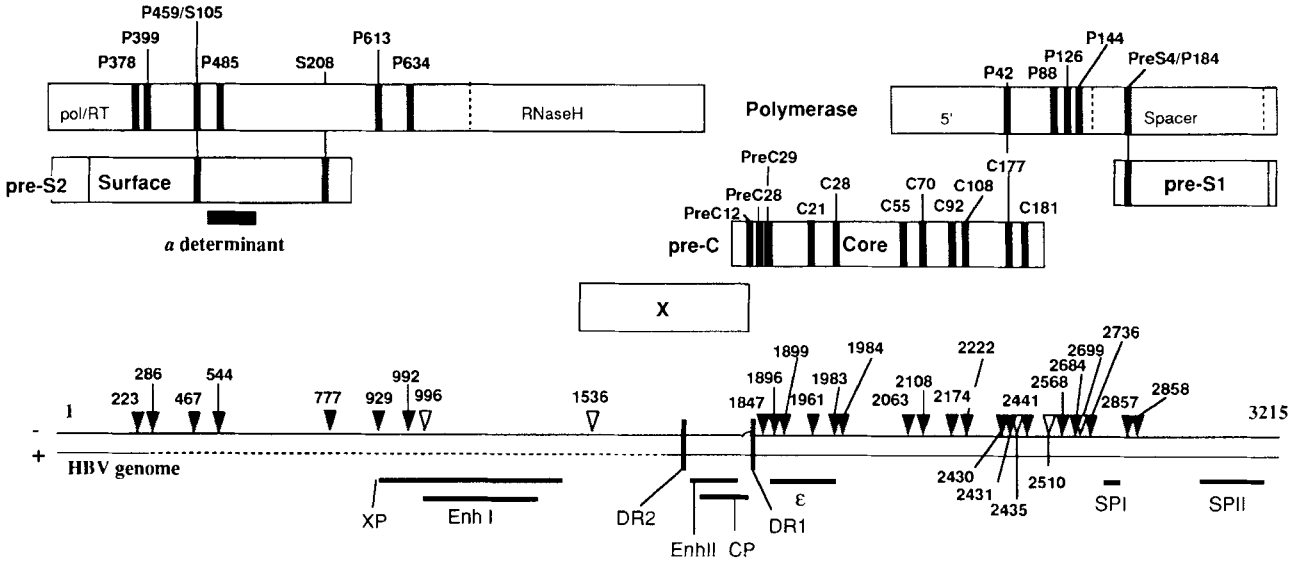

Fig. 1. Schematic demonstration of unique mutations in the patients with epidemic fulminant hepatitis. Four open reading frames of HBV genome are shown above the genome as rectangles, and locations of the regulatory elements are shown below. Vertical lines in each rectangle for open reading frames indicate the position of unique deduced amino acid mutations. Closed triangles above the genome indicate the position of missense nucleotide mutations, and open triangles indicate the position of silent mutations. Unique amino acid substitutions are located mainly in the pre-core/core gene and the polymerase gene. Abbreviations of regulatory element are as follows: Enh I, enhancer I; XP, X promoter; EnhII, enhancer II; CP, core promoter; SPI, surface promoter I; SPII, surface promoter II; DR1, direct repeat 1; DR2, direct repeat 2; ε, encapsidation signal ε; 5′, 5′ terminal binding peptide; pol/RT, DNA polymerase and reverse transcriptase; RNaseH, ribonuclease H.

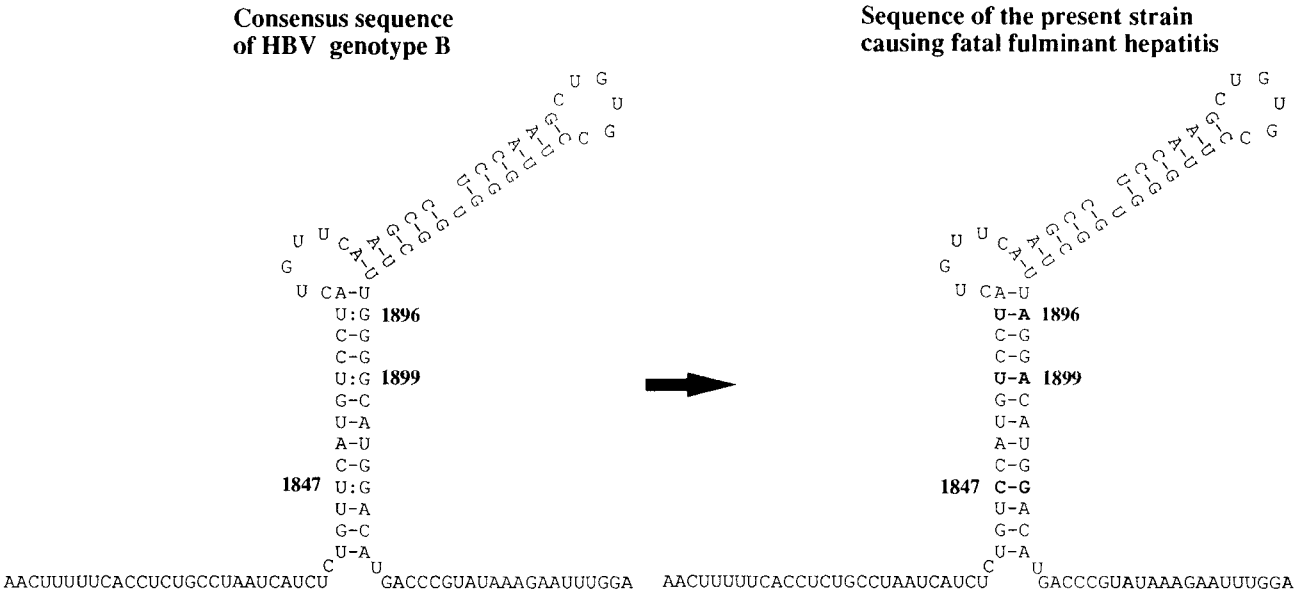

Fig. 2. Schematic demonstration of uncommon mutations in the encapsidation signal ε. Mutations at nt 1847, 1896, 1899 are postulated to make the stem-loop structure of HBV more suitable than that without these mutations.

at codons 28 and 29 in the pre-core region were common to the present strain and the reported strains.

DISCUSSION

The full-length genomes of an HBV strain associated with epidemic fulminant hepatitis were studied. Since two HBV genomes isolated from the different patients shared 99.6% identical nucleotide sequences, in spite of numerous unique mutations compared to the consensus sequence, these fatal fulminant hepatitis cases were likely to be an epidemic outbreak caused by a common source in a hemodialysis clinic. Unique mutations in the present strain causing fatal fulminant hepatitis B in unrelated individuals may contribute to the develop-

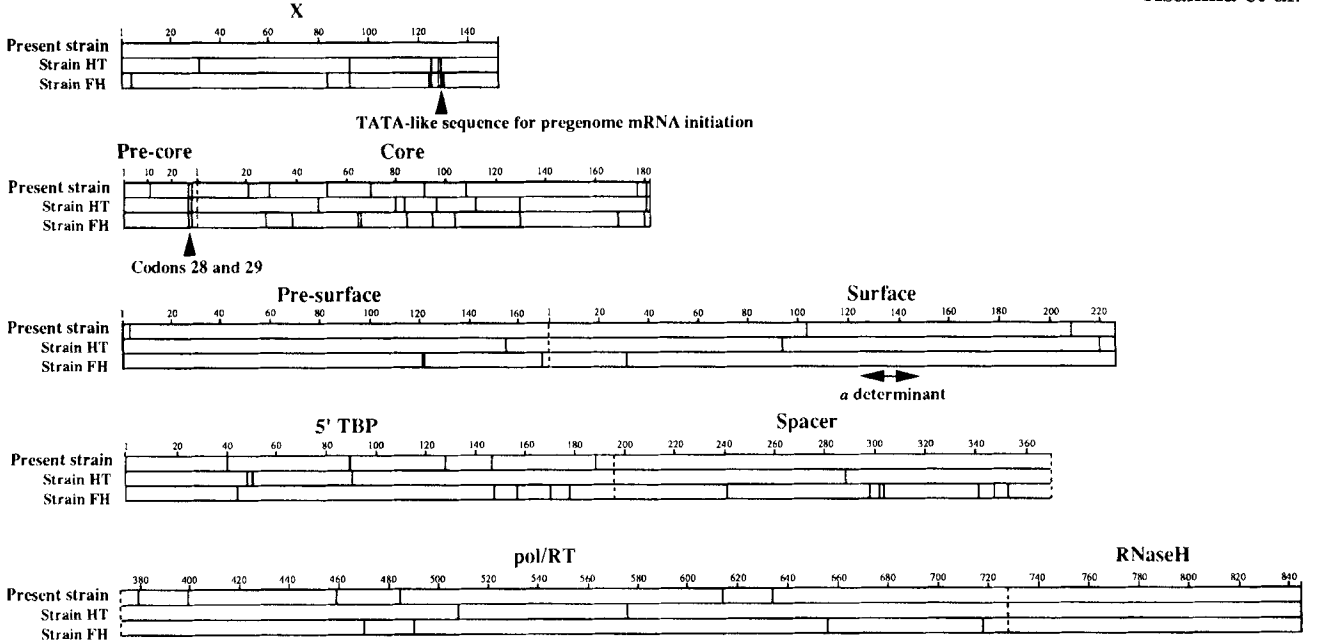

Fig. 3. Comparison of deduced amino acid residues for each open reading frame among present and previously reported HBV strains associated with epidemic fulminant hepatitis. The strain HT was reported to be associated with epidemic fulminant hepatitis in Japan [Ogata et al., 1993], and the strain FH was similarly reported in Israel [Hasegawa et al., 1994]. Vertical lines in each rectangle for open reading frames indicate the position of unique or uncommon deduced amino acid mutations. Mutations at codons 28 and 29 in the pre-core gene are commonly found in all three strains. Mutations in the TATA-like sequence for pregenomic mRNA initiation are found in both previously reported strains, but not in the present strain. Mutations found in three strains are mainly located in the pre-core/core gene and the polymerase gene. Abbreviations are as follows: 5′ TBP, 5′ terminal binding peptide; pol/RT, DNA polymerase and reverse transcriptase; RNaseH, ribonuclease H.

ment of fulminant hepatitis as a viral factor. Since the most dominant clone was determined in each patient by direct sequencing, the mutagenic effect of *taq* polymerase on the determination of HBV sequences could be negligible in the present analysis.

Unique nucleotide mutations in the regulatory elements were found mainly in the predicted stem-loop structure of the pregenome encapsidation signal sequence [Junker-Niepmann et al., 1990]. Nucleotide mutations at 1896 and 1899 were commonly observed in strain HT [Ogata et al., 1993], strain FH [Hasegawa et al., 1994], and the present strain, whereas no other common mutations were observed among these three strains. The mutation at nt 1899 and 1896 has been found frequently in subgenomically sequenced HBV isolates from patients with fulminant or fatal severe hepatitis [Liang et al., 1991; Omata et al., 1991; Yotsumoto et al., 1992; Ehata et al., 1993]. In contrast, they were infrequently found in self-limited acute hepatitis or nonfatal chronic hepatitis [Chuang et al., 1993; Horikita et al., 1994; Laskus et al., 1994; Lok et al., 1994], suggesting that these mutations are closely associated with severe hepatitis. In addition, a mutation was also observed at nt 1947 in our patients. Since mutations at nt 1847, 1896, and 1899 are postulated to make the stem-loop structure of HBV with these mutations more stable than that without these mutations (Fig. 2), precore defective mutants with these mutations are likely to have an advantage in viral replication [Tong et al., 1993; Laskus et al., 1994; Lok et al., 1994].

Many core mutations were found in the present study, as well as in strains HT and FH. In a previous subgenomic study, substantial numbers of unique substitutions clustering in this region were also found in fulminant and fatal severe hepatitis [Ehata et al., 1993], suggesting a possible association between mutations in the core region and severe liver injury. Since pre-core/core proteins are thought to contain target epitopes for CTL [Ferrari et al., 1987], HBV with mutations in this region could induce more intensive reaction of CTL, followed by severe hepatitis.

Eleven unique amino acid substitutions were found in the polymerase gene, especially in the 5′ terminal binding protein and the DNA polymerase-reverse transcriptase domains. Substantial numbers of mutations in the polymerase gene have also been reported in strains HT and FH [Ogata et al., 1993; Hasegawa et al., 1994]. In contrast, these mutations were rarely observed in patients with nonfatal chronic hepatitis B, as well as healthy HBV carriers (unpublished data). Therefore, these mutations seem to be associated with fulminant and severe hepatitis. The 5′ terminal protein is an important element for priming reverse transcription [Bartenschlager and Schaller, 1988; Weber et al., 1994], and the binding of DNA polymerase-reverse transcriptase to the encapsidation signal has been

shown to be responsible for both the packaging of pregenomic RNA and the synthesis of viral DNA [Pollack and Ganem, 1994]. Therefore, the polymerase gene itself is thought to have a critical role in a process of HBV replication, suggesting that mutations in this gene may affect viral replication, as well as mutations in the encapsidation signal. In addition, the enhancer I-X promoter element was overlapped in the polymerase gene, and two mutations were found in this element in the present study. Since these mutations may upregulate the X gene expression, a greater abundance of the transactivator also leads to increased HBV replication. Further investigations by a functional analysis are required to confirm the role of the polymerase gene mutations in fulminant hepatitis.

Six amino acid differences were found in the surface gene and in the polymerase gene between two HBV genomes isolated from two individuals in the present study. In particular, the differences were noted in the highly antigenic *a* determinant coding important epitopes for neutralizing antibodies. Since we have observed that mutations in this region serially occur during the course of HBV infection and that *a* determinant mutations are more frequently detected in patients who are seropositive for anti-HBs antibody (data in submission), the *a* determinant mutants may possibly be selected in the presence of anti-HBs antibody after the infection to the recipient. In fact, the *a* determinant mutations were only found in one patient who was positive for anti-HBs, but not the other patient who was negative. Therefore, these mutations themselves are unlikely to contribute to the development of fulminant hepatitis.

In contrast to strains HT and FH [Ogata et al., 1993; Hasegawa et al., 1994], no unique nucleotide mutation was found in the TATA-like sequence of the core promoter region that regulates the transcription of the pre-core messenger RNA. Recently, mutations in this region have been associated with the development of liver injury with decreased expression of HBeAg, as seen in pre-core stop codon mutants [Okamoto et al., 1994]. However, it is unlikely from the present findings that only these mutations themselves are associated with the development of fulminant hepatitis.

In conclusion, mutations in HBV genomes associated with epidemic fulminant hepatitis are described. They were mainly located in the polymerase gene, as well as in the pre-core/core gene, especially in the stem-loop structure of the pregenome encapsidation signal sequence.

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